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Progress Report
(Year 2: 1996)

"Mechanisms of Microgravity Effect on Vascular Function"

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Hypotheses

Hypothesis I. **HU Treatment decreases both maximal contractility and sensitivity to vasoconstrictor agonists.** Experiments carried out during year 1 addressed this hypothesis, and those results are summarized below.

HU treatment decreased the contractile response to 68 mM K^+ in abdominal aorta from Wistar (W) but not Sprague Dawley (SD) rats, compared to control. HU treatment also decreased the contraction to 68 mM K^+ in carotid arteries from both rat strains and in femoral arteries from W but not SD rats. HU treatment reduced the maximal response to norepinephrine in all arteries studied except the femoral artery from SD rats. HU treatment increased the contractile response of jugular vein from W rats to 68 mMK^+ but had no effect on that response in jugular vein from SD or in femoral vein from either strain of rat. HU treatment also had no significant effect on the maximal response of norepinephrine in veins, but there was a trend toward HU induced enhancement of contraction. These results demonstrate that HU treatment caused a nearly universal reduction in contractility in the arteries studied, but had either no effect or increased contractility in veins. Selected differences were identified between SD and W strains.

All work described in the present report was carried out using Wistar rats. Statistical analysis of concentration-response curves was carried out using

repeated measures, two way analysis of variance, followed by post hoc Scheffe's test for individual points between treatment groups. When only two means were compared, unpaired t test was used.

Another component of Hypothesis I was addressed by testing the effect of 20 day HU treatment on the vasuclar contraction to serotonin. HU treatment had no effect on the contraction of aorta (figure 1A), carotid artery (figure 2) or femoral artery (figure 3) to serotonin. There was a trend toward an HU-mediated reduction in the contraction of the jugular vein to serotonin (figure 4). However, this trend did not achieve significance, and HU treatment had no effect in the femoral vein (figure 5).

The lack of effect of HU treatment on the contraction of the aorta to serotonin was pursued in additional experiments. In each of these experiments, 4 rings were obtained from the aortas of control rats, and from HU rats. Two rings from each treatment group were contracted with serotonin and the other two, with norepinephrine. This allowed us to test for an HU treatment effect on the contraction to serotonin in rings from the same aorta in which HU treatment reduced contraction to norepinephrine. As shown in figure 1B, the contraction to norepinephrine was depressed by HU treatment. In contrast, HU treatment had no effect on the contraction to serotonin (figure 1C).

Hypothesis II. **Isolated blood vessels from HU rats will exhibit a generalized decrease in responsiveness to neurogenic stimulation, due to both presynaptic and postsynaptic mechanisms.** Ring segments of the caudal artery were mounted for the measurement of isometric contraction and contractile responses to nerve

terminal selective, electrical field stimulation were measured. Artery rings were subjected to 200 pulses of stimulation at 10 minute intervals and the frequency of stimulation was varied from 1 to 16 Hz. Thus, stimulation at 1 Hz required 200 seconds while that at 16 Hz required 12.5 seconds. The results are shown in figure 6. The strength of contraction in response to neurogenic stimulation increased with the frequency of stimulation in rings from both control and HU rats. There was a trend for the strength of contraction to be greater in the rings from HU rats. However, this was significant only at one Hz. Moreover, when the stimulation was repeated at one Hz, the difference between control and HU was no longer significant. Thus, HU treatment appears to have either no effect on contraction to neurogenic stimulation, or to transiently increase contraction only at the lowest frequency studied.

Hypothesis III. **Both endothelium-dependent and -independent vasodilation are not altered by HU treatment.** Several experiments were carried out during Year 2 to assess the role of endothelium in the effect of HU treatment on the response to norepinephrine. As shown in figure 7, HU treatment significantly depressed the maximal contraction to norepinephrine in abdominal aorta rings with endothelium either intact or denuded. Similar results were obtained in femoral arteries (figure 8). However, different results were obtained in the carotid artery and femoral vein. In the carotid artery (figure 9), with intact endothelium, HU treatment reduced the contractile response to norepinephrine, and that reduction was significant between 1 and 100 nM, and at 300 μM norepinephrine. In contrast, in rings in which the endothelium had been mechanically removed, HU treatment had a small but significant effect only at 1

nM norepinephrine. At all higher concentrations the norepinephrine concentration response curves in vessels from control and HU treated rats were completely superimposable. These experiments show greater variability, as evidenced by larger error bars, compared to our previous work, reported in the progress report for year 1. In all previous experiments, 4 artery rings per treatment group per experiment were used. In these experiments, 2 artery rings were used per group per experiment in order to study the effects of norepinephrine in endothelium intact and endothelium denuded vessels from the same animals.

It was technically difficult to mechanically remove the endothelium from the femoral vein. Thus, when this vessel was studied, endothelium intact rings were exposed to L-nitroarginine methyl ester (L-NAME) to inhibit the synthesis of nitric oxide, the vasodilator substance released from the endothelium. The results are shown in figure 10. Hu treatment tended to decrease the contraction to norepinephrine, and this trend became significant at 300 μM norepinephrine. However, in L-NAME-treated tissues, the HU effect was abolished and the control and treated norepinephrine concentration-response curves became superimposed.

In order to assess the possible effect of HU treatment on endothelial mechanisms, a different experimental design was used. Artery rings were first contracted by incremental additions of phenylephrine to the bathing medium. After exposure to the highest concentration of phenylephrine used, acetylcholine was added incrementally to assess endothelium-dependent, acetylcholine-mediated

relaxation. Both the phenylephrine concentration contraction-response curves and the acetylcholine relaxation-response curves in carotid artery are shown in figure 11. HU treatment depressed the contractile response of the carotid artery to phenylephrine (figure 11A). In order to compare acetylcholine relaxation curves in control vs. HU carotid arteries, the magnitude of phenylephrine contraction was normalized between the treatment groups by expressing these contractions as 100%. HU pretreatment caused an increase in the sensitivity of the carotid artery to the relaxing effects of acetylcholine (figure 11B). Thus, most of the acetylcholine relaxation-response curve in tissues from HU treated animals was in a concentration range 7-10-fold below that observed in control tissues.

After exposure to the maximal concentration of acetylcholine, the tissue baths containing these same carotid artery rings were drained and refilled twice with fresh Krebs' solution. Subsequently, the tissues were contracted once again with phenylephrine and at maximal contraction, were exposed to increasing concentrations of Na^+ nitroprusside over a concentration range of 0.1 nM to 3 μM. It can be seen in figure 11C that HU treatment had no effect on the Na^+ nitroprusside induced relaxation. These results with acetylcholine and sodium nitroprusside show that HU treatment increases the sensitivity of the carotid artery to endothelium-dependent relaxation (acetylcholine), but has no effect on endothelium-independent relaxation (Na^+ nitroprusside).

Similar experiments were carried out using the femoral artery. Artery rings from control and HU-treated rats were contracted with phenylephrine, followed by

relaxation with acetylcholine (figure 12). HU treatment did not effect the contractions to phenylephrine. Moreover, it is clear that HU treatment had no effect on the sensitivity of the femoral artery to the relaxing effects of acetylcholine(figure 12B). Similarly, HU treatment had no effect on the femoral artery relaxation to sodium nitroprusside (figure 12C).

The same experimental approach was used to analyze the effects of HU treatment on the abdominal aorta, with surprising results. As shown in figure 13A, HU treatment caused the expected reduction in the contraction to phenylephrine. However, HU treatment had no effect on the relaxation to acetylcholine (figure 13B). In contrast, when phenylephrine-contracted aorta rings were exposed to sodium nitroprusside, it was found that HU treatment reduced the sensitivity of the abdominal aorta rings to this endothelium-independent relaxing agent.

In summary, HU treatment increased the sensitivity of the carotid artery, but not the aorta or femoral artery, to the relaxing effects of acetylcholine. On the other hand, HU treatment decreased the sensitivity of the abdominal aorta, but not the carotid or femoral arteries, to the relaxing effects of sodium nitroprusside.

In order to gain further insight into possible effects of HU treatment on endothelial function, protein mass of nitric oxide synthase was measured in carotid and femoral arteries using Western blot. The experiment was performed twice using tissues pooled from 3-6 control and HU treated rats per experiment. As shown in figure 14, HU treatment increased the protein mass of nitric oxide synthase in both the

carotid and femoral arteries. This could explain the important role of endothelium in the reduced magnitude of contraction of the carotid artery to norepinephrine as well as the increased sensitivity of this vessel to the relaxing effects of acetylcholine. However, presently there is no explanation for the lack of HU effect on these parameters in the femoral artery which also exhibited an increase in nitric oxide synthase protein mass.

Hypothesis IV. **HU - Induced changes in vascular function follow the same time course for development and recovery that is found for skeletal muscle.** In order to test this hypothesis, experiments to date have involved measuring the weights of soleus muscles for comparison to measurements of abdominal aorta carotid and femoral arteries to norepinephrine, and measurement of the relaxation response to acetylcholine. Soleus muscle weights are shown in Table 1. HU treatment caused a significant reduction in soleus muscle weight at 20 and 7 days of treatment but not at 3 days of treatment.

Figures 15 - 18 show the effects of 1 to 20 day HU treatment on contractile responses of abdominal aorta to both 68 mM K^+ and norepinephrine. HU treatment caused a significant reduction in contraction to both stimuli at 20, 7, and 3 day, but not 1 day, HU treatment.

Figures 19 - 22 show the effects of HU treatment on contractile responsive carotid artery to both 68mM K^+ and norepinephrine. HU treatment caused a significant reduction in contraction to both stimuli at 20, 7, 3, and 1 day, HU treatment.

Figures 23 - 25 show the effects of 3 to 20 day HU treatment on the contractility of the femoral artery to both 68mMK^+ and norepinephrine. HU treatment reduced the contractility of the femoral artery only at 20 days of treatment.

The time course of HU treatment effects on relaxation responses to acetylcholine and Na^+ nitroprusside has also been explored. Time course experiments to date include only the carotid and femoral arteries. The results of phenylephrine contraction followed by either acetylcholine- or sodium nitroprusside-mediated relaxation at 20 days HU treatment are described earlier in this report and are shown in figure 11. As shown in figure 26, 7 day HU treatment reduced the contractile response of the carotid artery to phenylephrine. However, the 7 day HU treatment had no effect on relaxations elicited by either acetylcholine or sodium nitroprusside. The effects of 3 day HU treatment on phenylephrine contraction followed by either acetylcholine or sodium nitroprusside relaxation are shown in figure 27. Three day HU Treatment had no effect on any parameter studied.

The effect of 20 day HU Treatment on the femoral artery contraction to phenylephrine followed by relaxation to either acetylcholine or sodium nitroprusside are shown in figure 28. HU treatment had no effect.

In summary, there appears to be no correlation between the effects of HU treatment on the rate of soleus muscle atrophy compared to vascular contraction or relaxation. The soleus muscles from HU rats weighed less than the paired controls after both 20 and 7 days of treatment. In contrast, HU treatment decreased contraction to norepinephrine after 3 days in aorta and after just 1 day in carotid artery, treatment periods when the soleus muscle was unaffected. On the other hand, only 20-day HU treatment decreased contraction to norepinephrine in the femoral artery. Similarly acetylcholine-mediated relaxation of the precontracted carotid artery was seen only after 20 days of HU treatment.

OVERALL SUMMARY OF OBSERVATIONS

1. While HU treatment depressed the maximal contractile response to norepinephrine, it had no effect on that to serotonin.

2. HU treatment has either a small transient, or no effect on neurogenic contraction of rat tail artery.

3. The HU-induced reduction of contractility in aorta and femoral artery was not affected by endothelium removal.

4. The HU-induced reduction of contractility was abolished by mechanical removal of endothelium in the carotid artery, or by L-NAME treatment in the femoral vein.

5. 20-Day HU treatment increased the sensitivity of contracted carotid artery to acetylcholine-mediated relaxation but had no effect on the sensitivity of that mediated by Na^+ nitroprusside.

6. 20-Day HU treatment had no effect on either acetylcholine- or Na^+ nitroprusside-mediated relaxation in femoral artery.

7. 20-Day HU treatment had no effect on acetylcholine relaxation in aorta, but decreased the sensitivity of this vessel to Na^+ nitroprusside-mediated relaxation.

8. 20-day HU treatment increased the protein mass of nitric oxide synthase in both carotid and femoral arteries.

9. 20- 7- and 3-, but not 1-Day HU treatment decreased the maximal contraction to norepinephrine in aorta.

10. 20-, 7-, 3- and 1-Day HU treatment decreased the maximal contraction to norepinephrine in carotid artery.

11. Only 20-day HU treatment decreased maximal contraction to norepinephrine in femoral artery.

12. Only 20-day HU treatment increased the sensitivity of the contracted carotid artery to acetylcholine.

The factors modulated by HU treatment are complex and nonuniform in the vasculature. HU treatment appears to have an inhibitory effect on vascular smooth muscle contractile mechanisms in aorta and femoral artery, but may depress contraction in carotid artery by an additional endothelium-dependent mechanism. HU treatment selectively decreased the vascular smooth muscle sensitivity of aorta to the endothelium-independent vasodilator, Na^+ nitroprusside. Since Na^+ nitroprusside spontaneously releases nitric oxide, this HU effect could have masked a possible increase in sensitivity of the aorta to the nitric oxide-mediated relaxing effect of acetylcholine. HU treatment increased the sensitivity of the carotid, but not the femoral artery or aorta to acetylcholine. Both carotid and femoral arteries from HU rats exhibited increased protein mass of nitric oxide synthase. All three vessels from HU rats exhibited a depressed contractile response to norepinephrine, but not to serotonin. In conclusion, HU treatment may affect smooth muscle contractile mechanisms, endothelial dilator mechanisms and membrane receptor mechanisms in blood vessels. HU effect on these parameters varied among the vessels studied and exhibited a differential time course for the onset of effect.

PLANS FOR THE NEXT YEAR

Each remaining task of the project is listed below, followed by the number of the specific aim to which it is related in the original proposal.

1. Characterize the effects of HU treatment on the middle cerebral artery (I).

2. Subject rings of jugular vein to electrical field stimulation to study the effect of HU treatment on beta adrenoceptor-mediated neurogenic vasodilation (II).

3. Subject the isolated, perfused mesenteric vasculature to neurogenic stimulation to study the effect of HU treatment on vasodilation by capsaicin-sensitive sensory nerves (II).

4. Compare the vasodilator responses of aorta, carotid and femoral arteries to Na^+ nitroprusside and calcitonin gene-related peptide (CGRP) and the dilator responses of jugular vein to isoproterenol, a beta adrenergic agonist, in control and HU treated rats (III).

5. Determine the time course of recovery from 20-day HU treatment of the most salient vascular parameters and compare this to the time course of recovery to normal weight by the soleus muscle (IV).

6. Determine the effect of HU treatment on the cellular mobilization of calcium from the external medium and from intracellular stores, with simultaneous measurement of contraction in rings of aorta, carotid, and femoral artery (V_A).

7. Use two-dimensional gel electrophoresis to screen for specific proteins in vascular smooth muscle that may be down-regulated by HU treatment (V_A).

8. Determine the effect of inhibitors of phospholipase C, protein kinase C and voltage-regulated calcium channels on the contraction of vascular rings in order to identify effects of HU treatment on second messenger signaling pathways (V_B).

9. Compare the effects of indomethacin (prostaglandin synthesis inhibitor) and L-NAME (nitric oxide synthase inhibitor) on acetylcholine-mediated relaxation in carotid artery to assess the effects of HU treatment on prostaglandin- versus nitric oxide-dependent vasodilator mechanisms (V_D).

10. Compare the vasodilator responses of aorta to Na^+ nitroprusside versus 8-bromo-cyclic guanosine monophosphate to determine further the site of desensitization by HU treatment of the aorta to endothelium-independent vasodilators (V_D).

Table 1. Weights (grams) of soleus muscles from control and HU rats, presented as means ± S.E.M.

Treatment Duration	Control	HU	N
3 - Day	0.12 ± 0.06	0.10 ± 0.05	4
7 - Day	0.11 ± 0.04	0.07 ± 0.006*	8
20 - Day	0.14 0.009	0.10 ± 0.01*	6

* Significantly different from control; $P<0.05$ (Unpaired test)

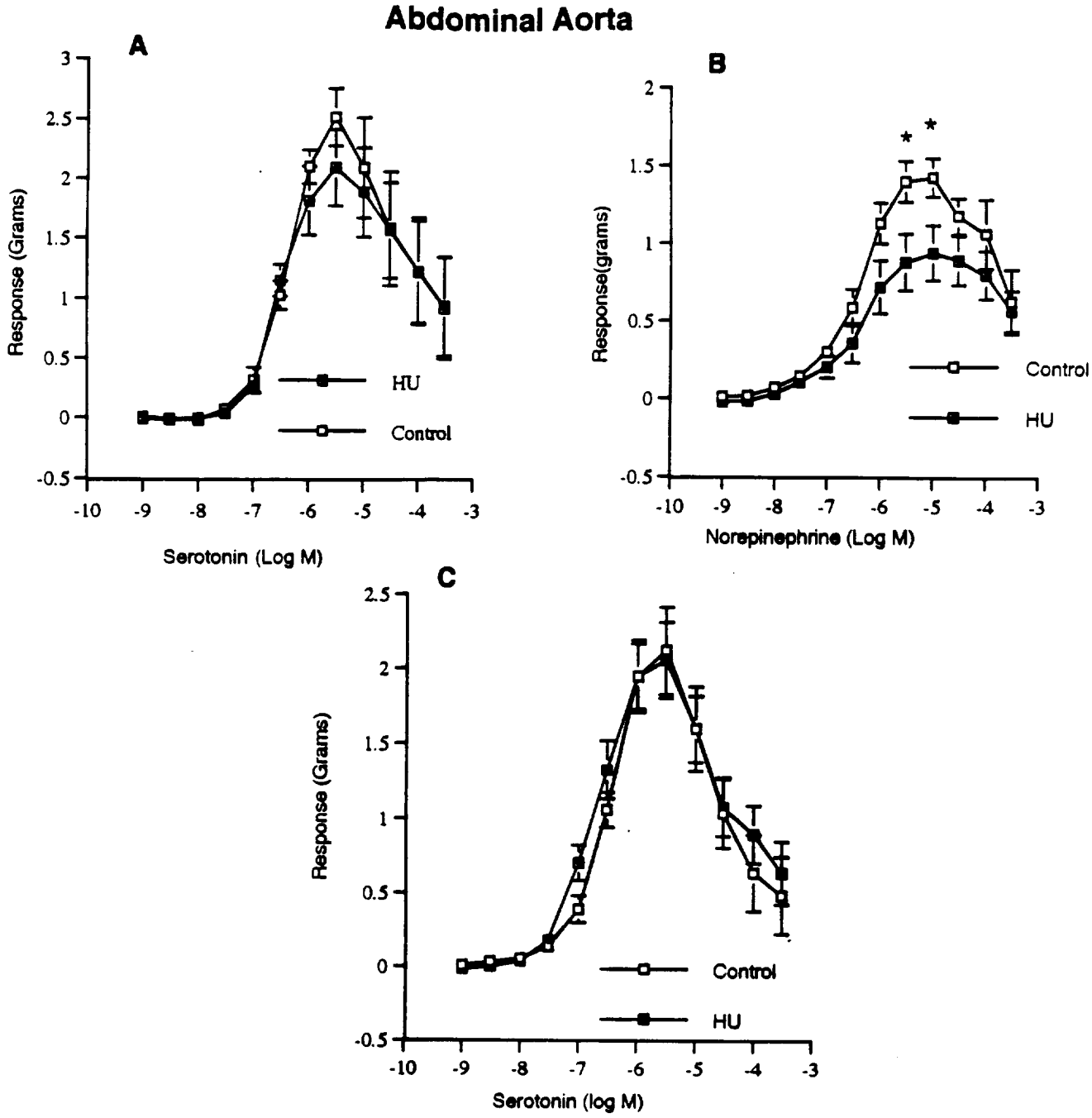


Figure 1. Concentration-response curves for the contractile effects of serotonin and norepinephrine in abdominal aorta from control and 20-day HU rats. Panel A represents results based on the use of 4 rings per treatment group per experiment. The data represented in panels B and C were obtained by using two rings each from control and HU aortas for serotonin and the other two rings per treatment group per experiment for norepinephrine. This allowed assessment of the HU effect on the response to serotonin in parallel with that to norepinephrine in tissues from the same animals. *, $P<0.05$, $N=5$.

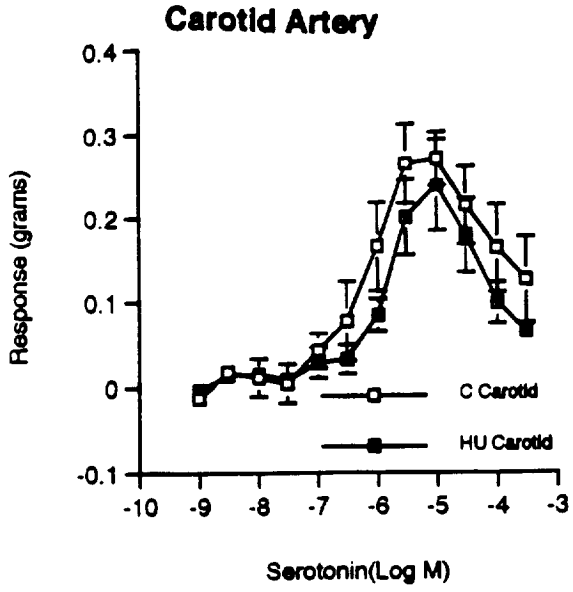


Figure 2. Concentration-response curves for the contractile effects of serotonin in carotid artery from control and 20-day HU rats. N=5.

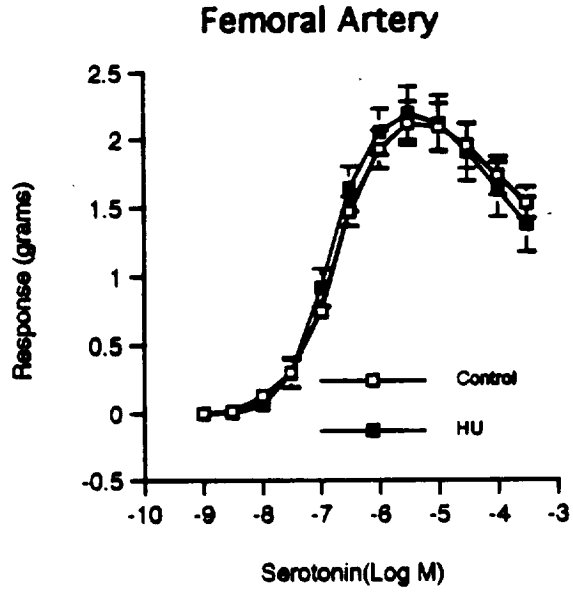


Figure 3. Concentration-response curves for the contractile effects of serotonin in femoral artery from control and 20-day HU rats. N=6.

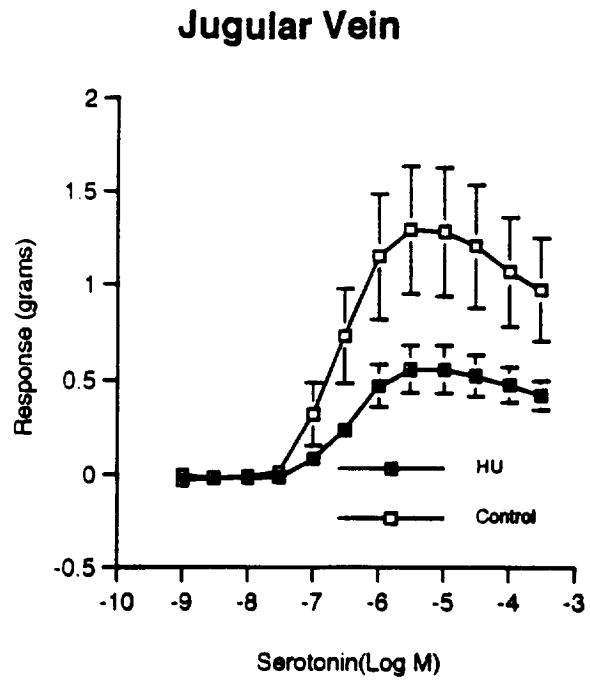


Figure 4. Concentration-response curves for the contractile effects of serotonin in jugular vein from control and 20-day HU rats. N=5.

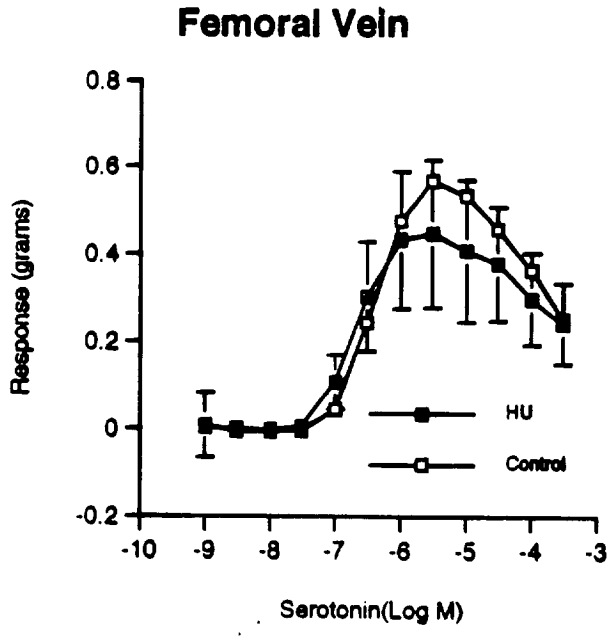


Figure 5. Concentration-response curves for the contractile effects of serotonin in femoral vein from control and 20-day HU rats. N=5.

Tail Artery

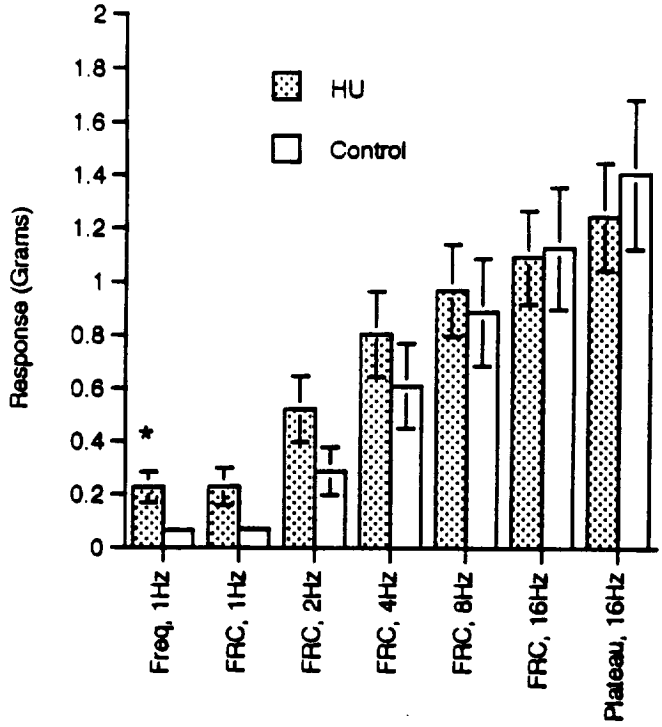


Figure 6. Contractile responses of tail artery rings from control and 20-HU rats to electrical field stimulation. Artery rings were subjected to 200 pulses of stimulation at 10 minute intervals and the frequency of stimulation was varied from 1 to 16 Hz. The last stimulation at 16 Hz was continued beyond 200 pulses until the contractile response reached plateau. *, $P<0.05$, $N=5$.

Abdominal Aorta

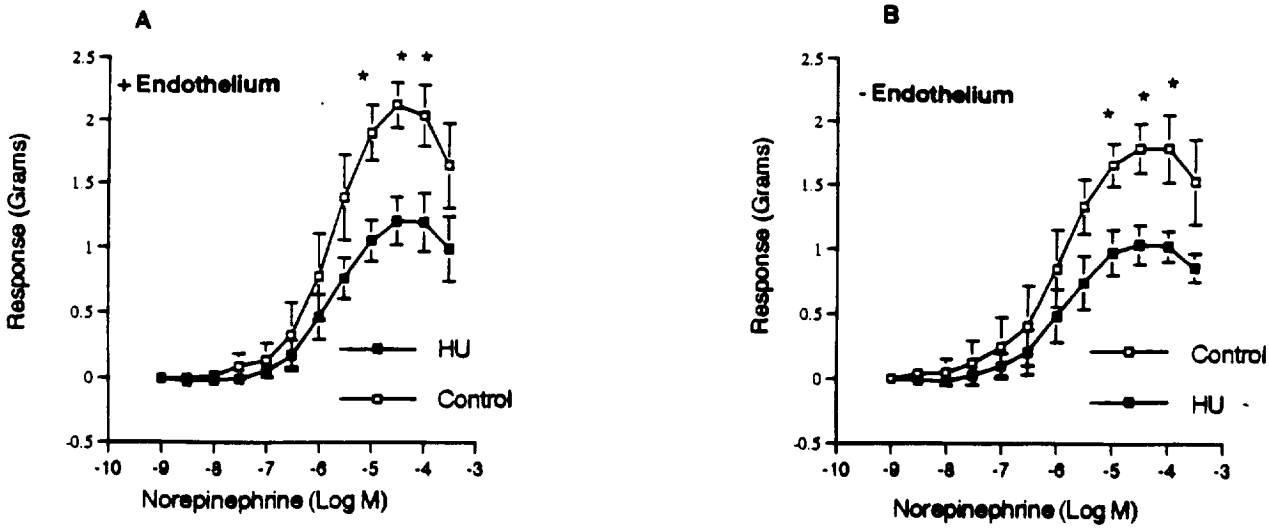


Figure 7. Concentration-response curves for the contractile effects of norepinephrine in abdominal aorta from control and 20-day HU rats. Two of 4 rings from each aorta were mechanically de-endothelialized. Panels A and B show results from endothelium-intact and denuded rings, respectively. *, $P<0.05$, N=5.

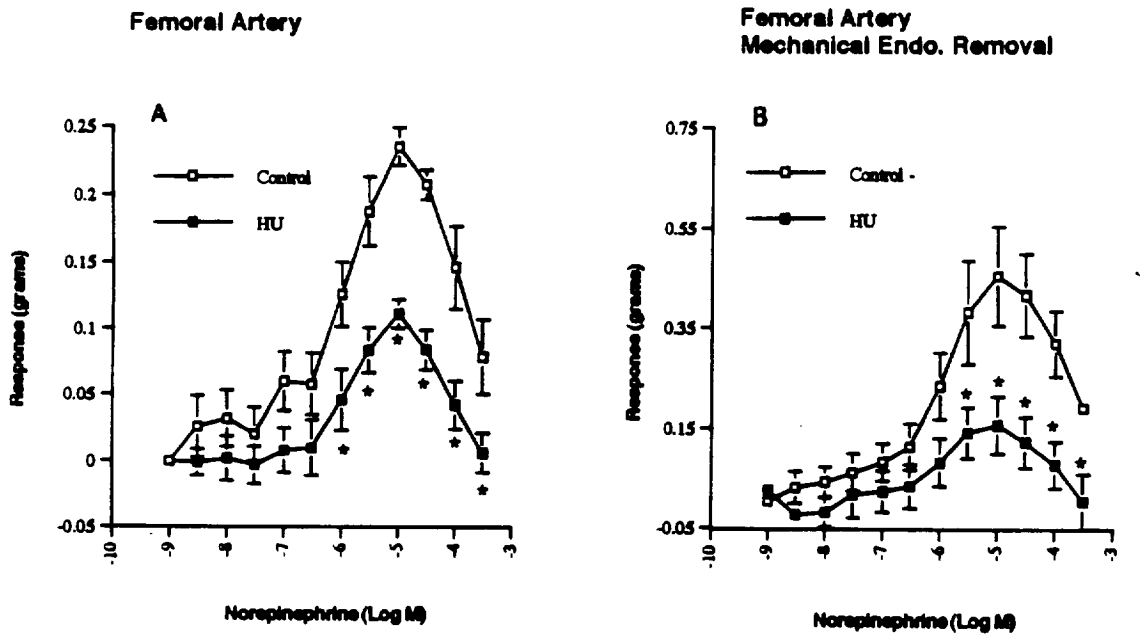


Figure 8. Concentration-response curves for the contractile effects of norepinephrine in femoral arteries from control and 20-day HU rats. Two of 4 rings from each animal were mechanically de-endothelialized. Panels A and B show results from endothelium-intact and denuded rings, respectively. *, $P<0.05$, N=5 and 6 for panels A and B respectively.

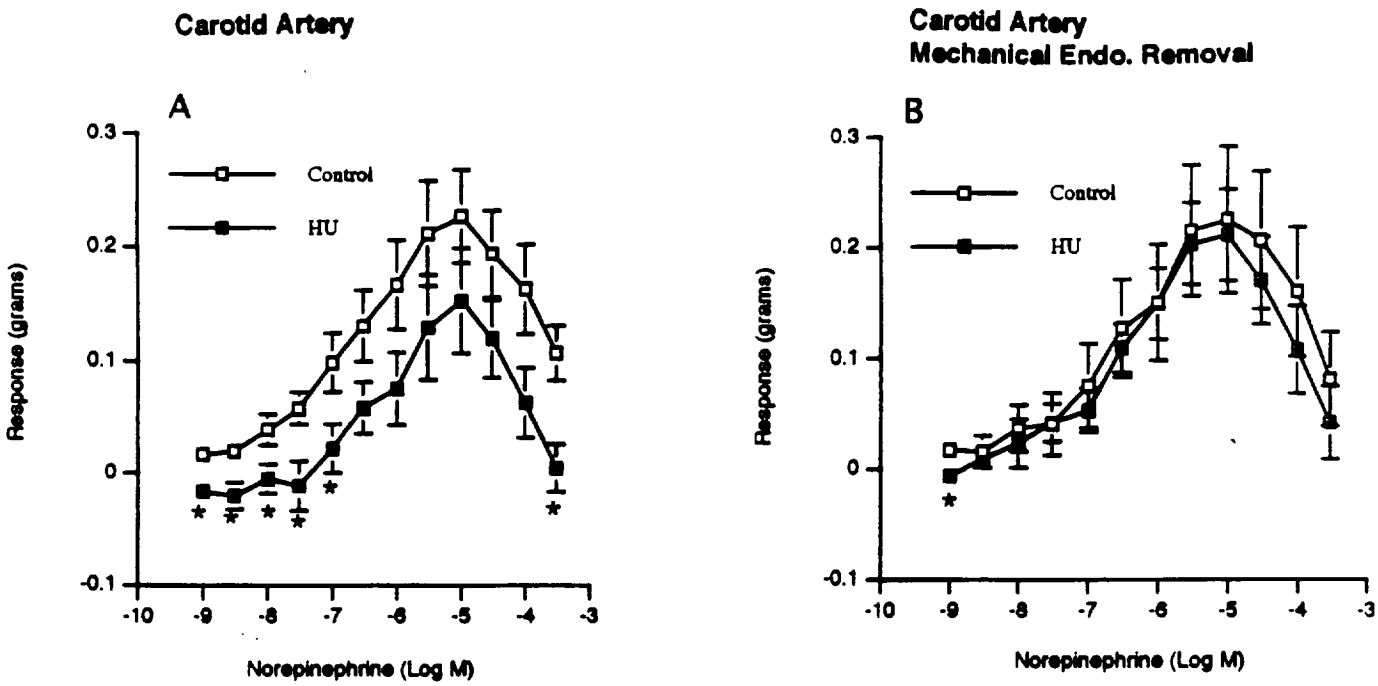


Figure 9. Concentration-response curves for the contractile effects of norepinephrine in carotid artery from control and 20-day HU rats. Two of 4 rings from each animal were mechanically de-endothelialized. Panels A and B show results from endothelium-intact and denuded rings, respectively. *, $P<0.05$, N=10 and 11 for panels A and B, respectively.

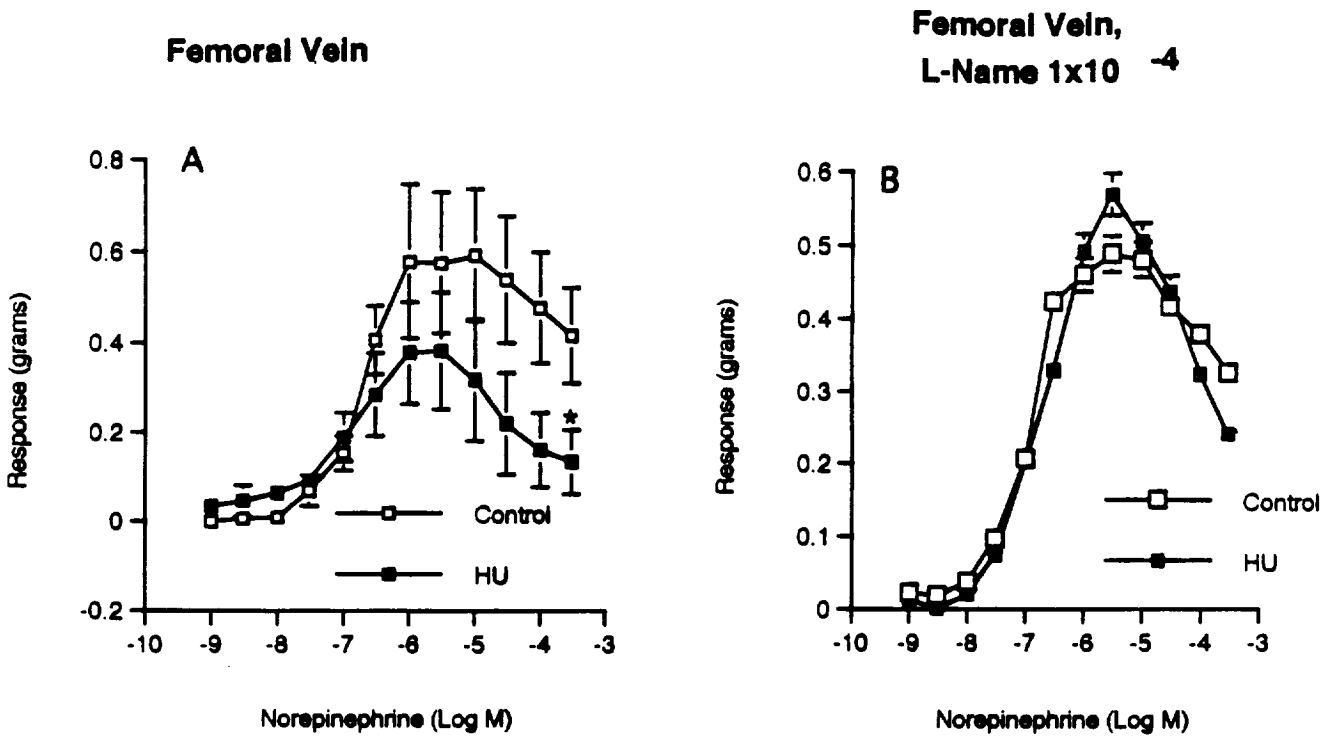


Figure 10. Concentration-response curves for the contractile effects of norepinephrine in femoral vein from control and 20-day HU rats. Two of 4 rings from each animal were exposed to L-NAME to block nitric oxide synthase during the experiment. Panels A and B show results from control and L-NAME-treated rings, respectively. *, $P<0.05$, $N=8$.

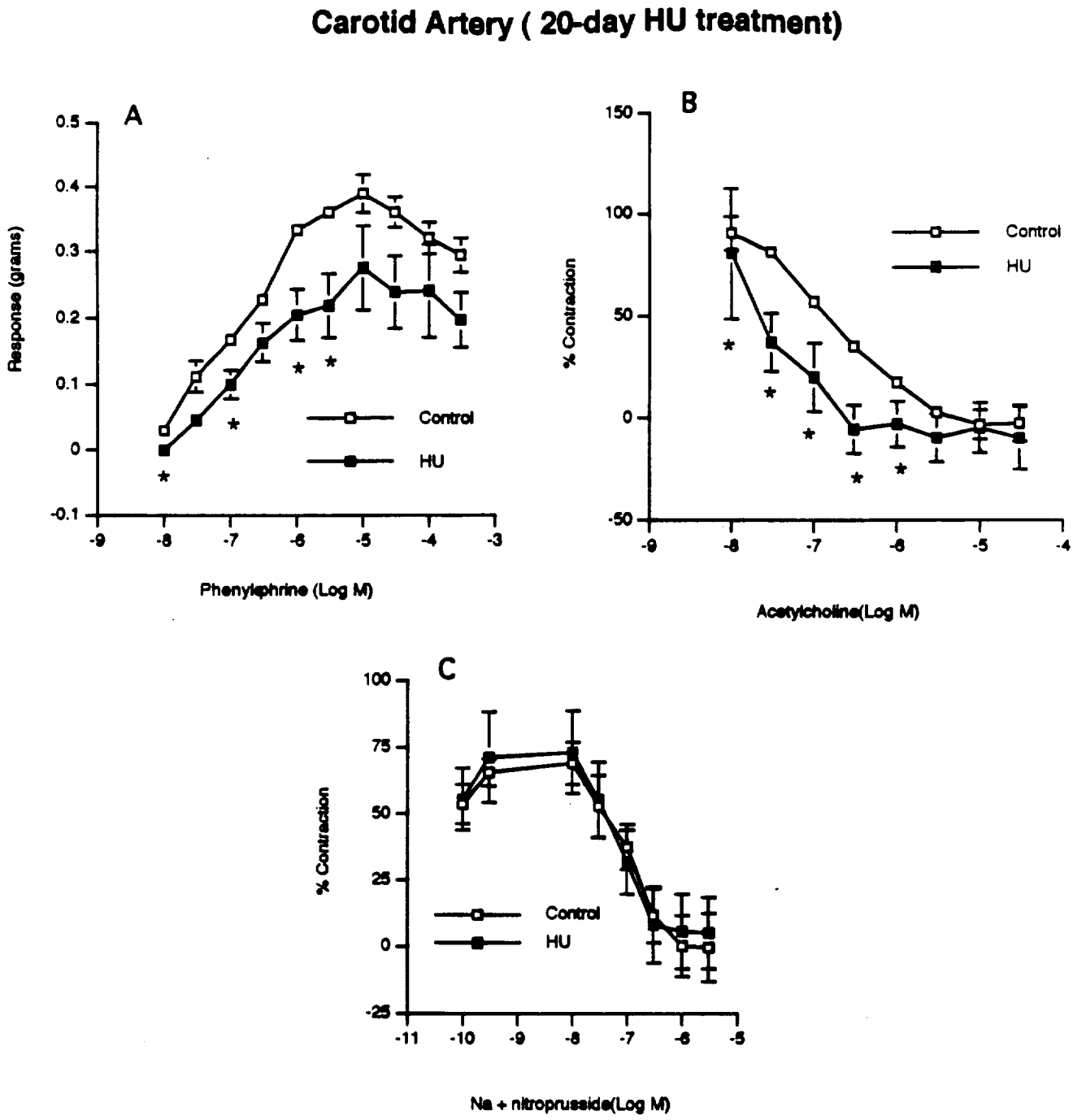


Figure 11. Concentration-response curves for the contractile effect of phenylephrine (panel A) and the relaxation effects of acetylcholine (panel B) and Na^+ nitroprusside (panel C) in carotid arteries from control and 20-day HU rats. *, $P<0.05$, N=4.

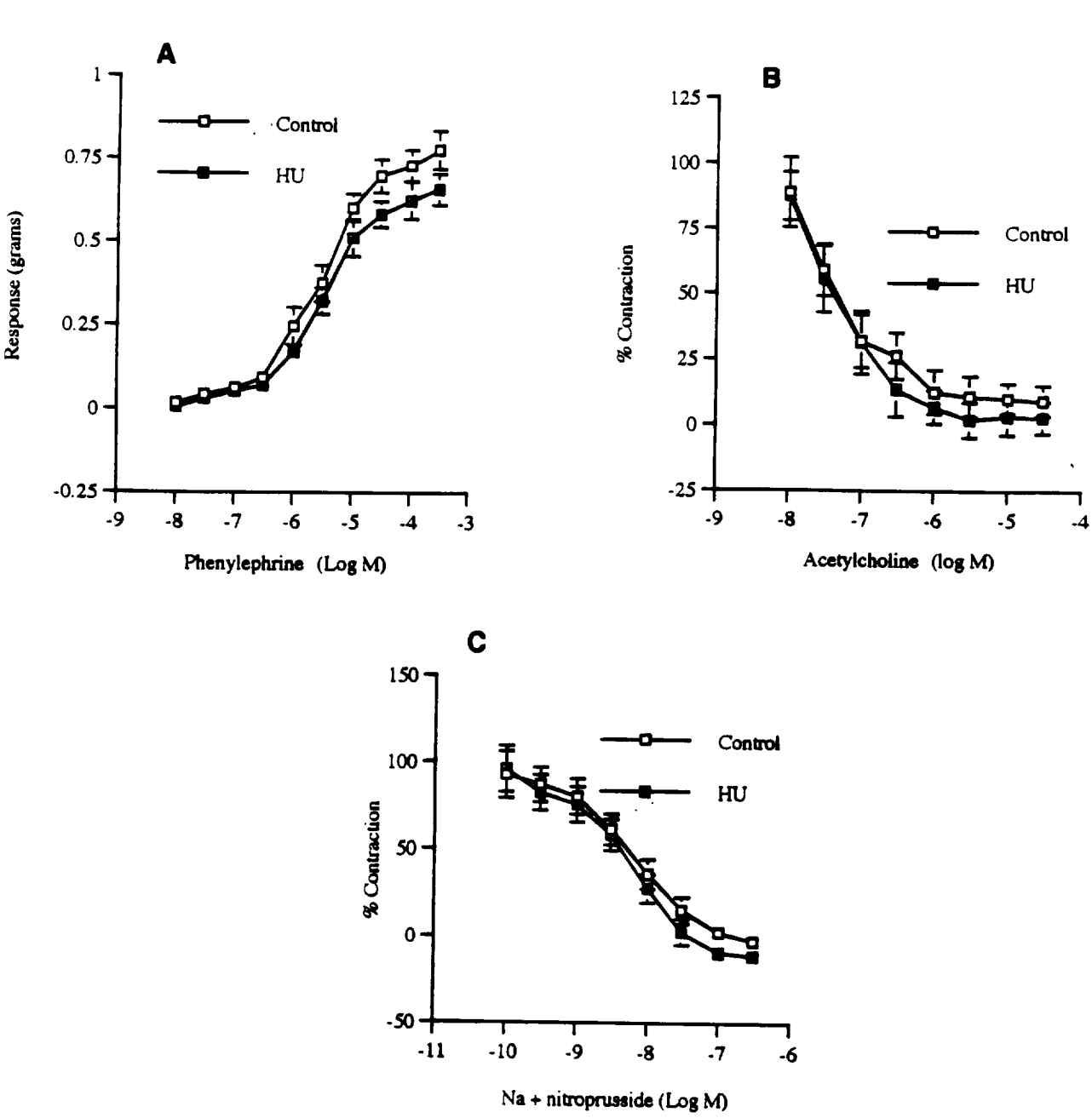


Figure 12. Concentration-response curves for the contractile effect of phenylephrine (panel A) and the relaxation effects of acetylcholine (panel B) and Na^+ nitroprusside (panel C) in femoral arteries from control and 20-day HU rats. N=5.

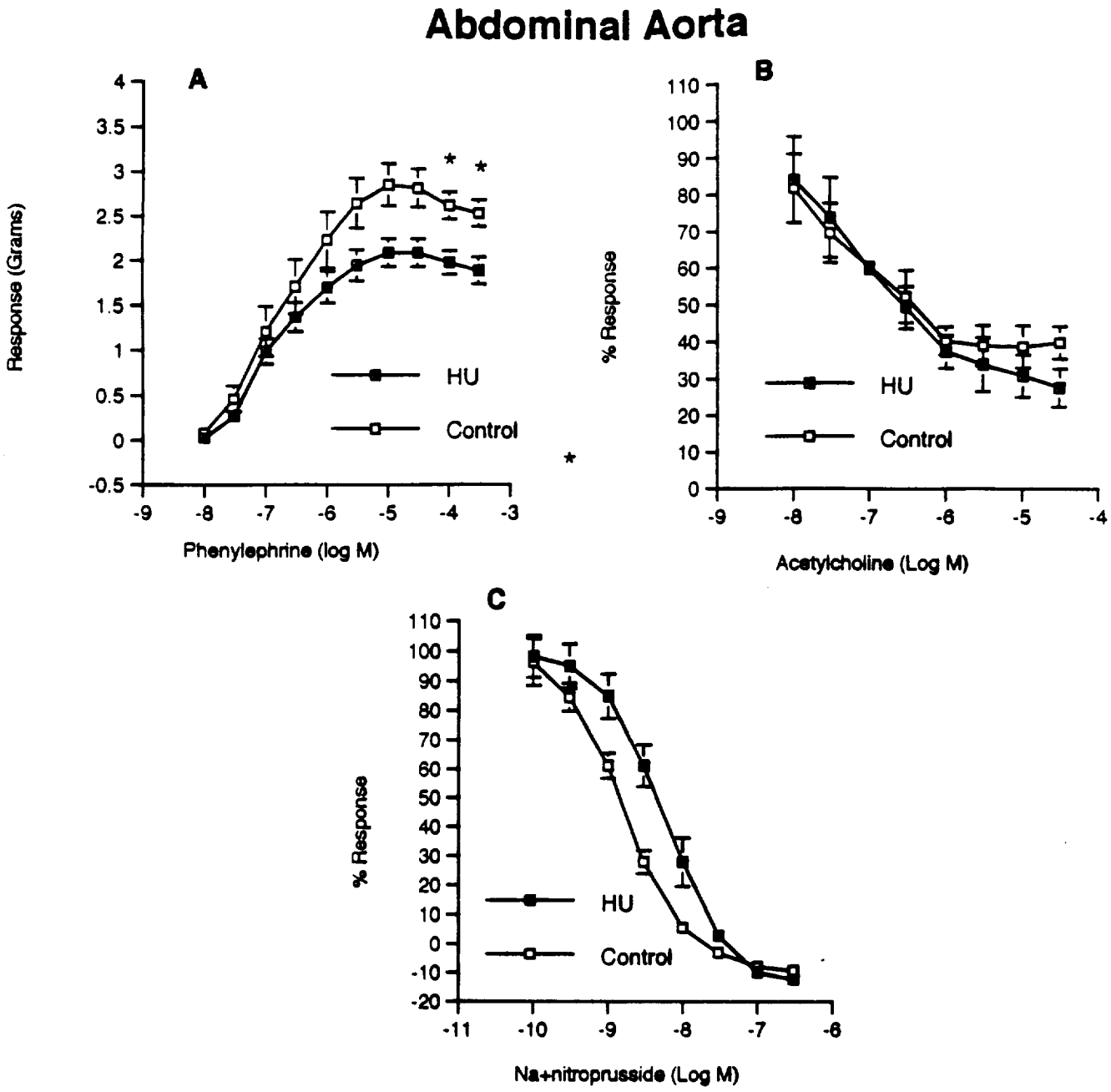
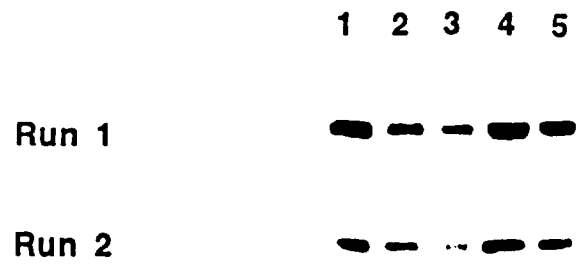


Figure 13. Concentration-response curves for the contractile effect of phenylephrine (panel A) and the relaxation effects of acetylcholine (panel B) and Na^+ nitroprusside (panel C) in abdominal aorta from control and 20-day HU rats. *, $P<0.05$, $N=4$.



Relative Density readings	Lanes 1	2	3	4	5
Run 1	5489	3602	3051	5356	4542
Run 2	3122	2877	2387	4191	3070

Figure 14. Western blots of protein mass of endothelial nitric oxide synthase (eNOS). Proteins were from samples pooled as follows: lanes 1 and 2: carotid arteries from 3 20-day HU rats; lane 3: carotid arteries from 3 control rats; Lane 4: femoral arteries from 6 20-day HU rats; lane 5: femoral arteries from 6 control rats. Runs 1 and 2 are two independent Western blots from the pooled samples listed above.

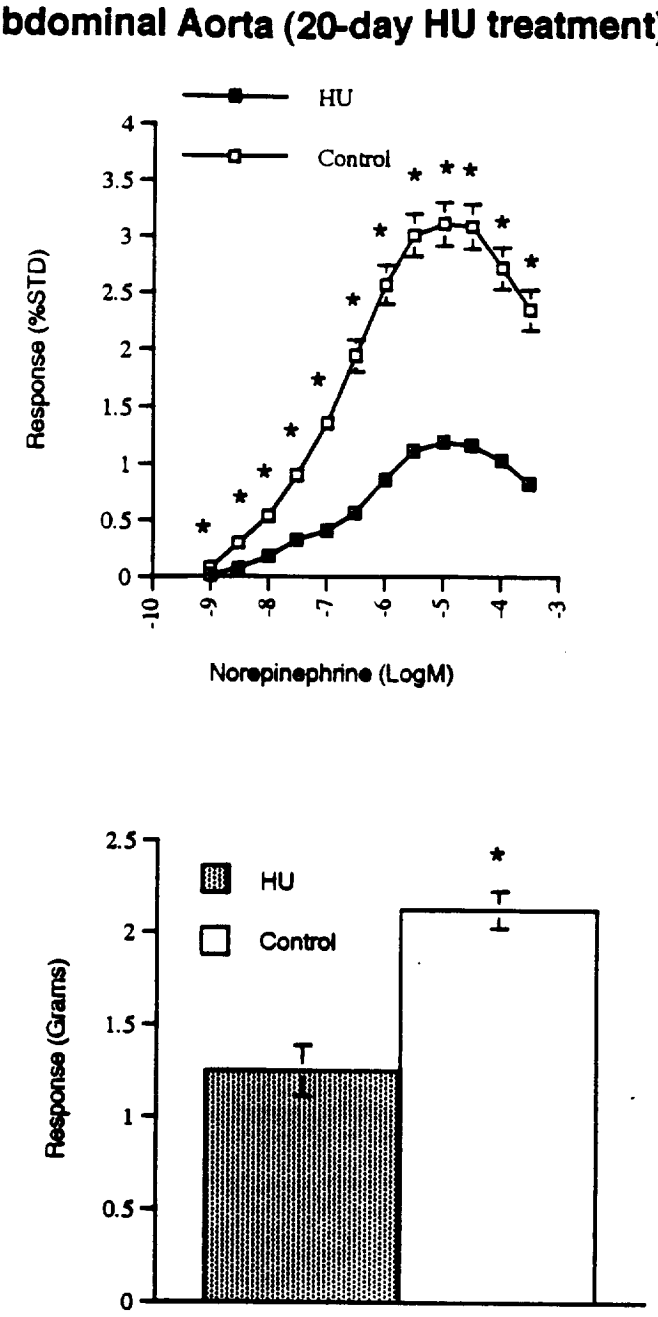


Figure 15. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in abdominal aorta from control and 20-day HU rats. *, $P<0.05$, N=5.

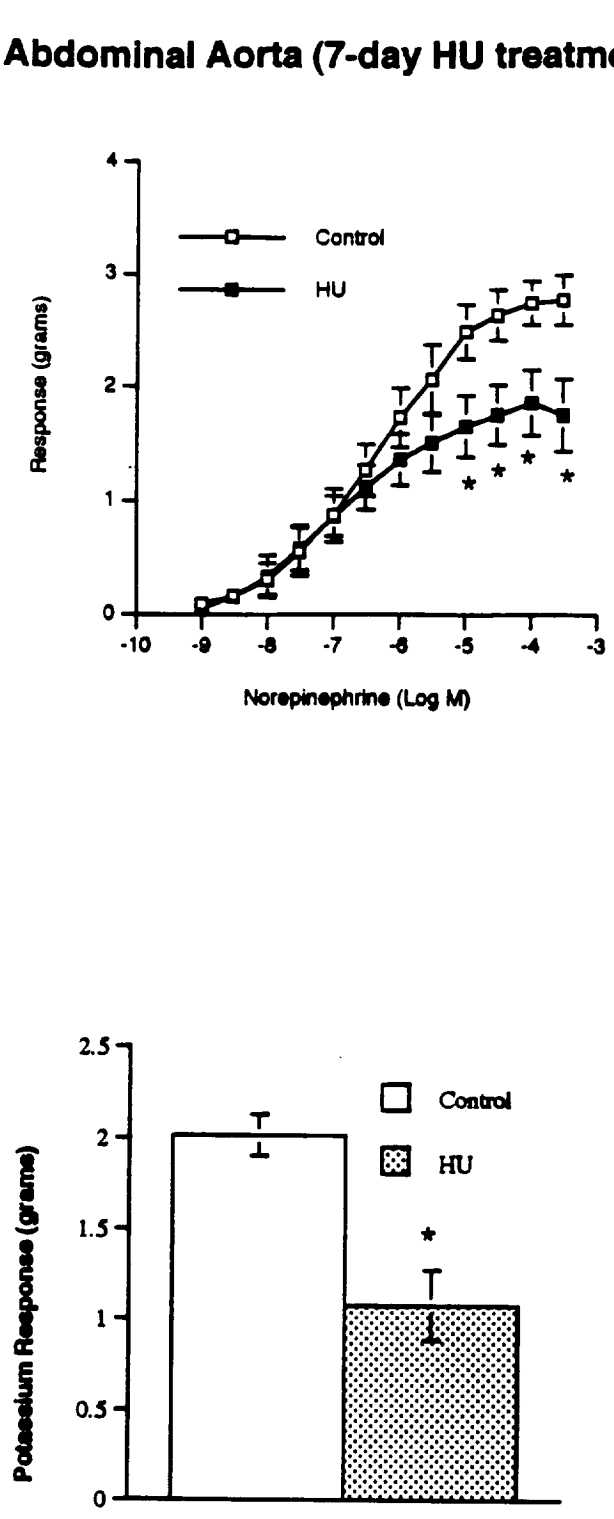
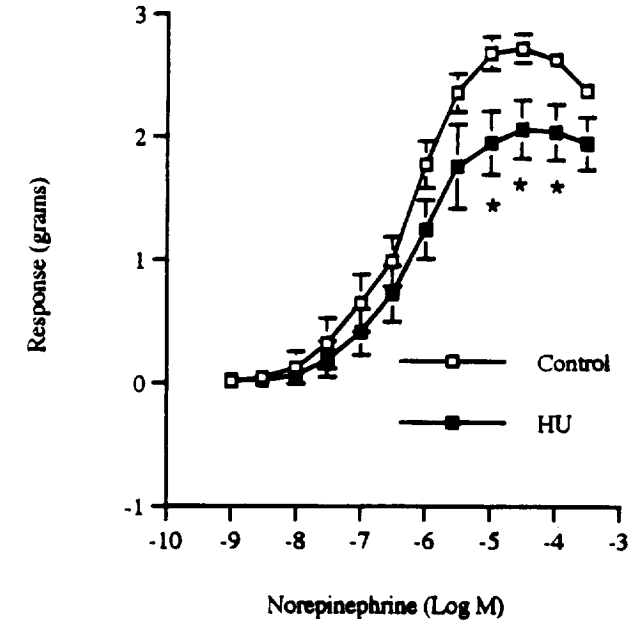


Figure 16. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) *in abdominal* aorta from control and7-day HU rats. *, P<0.05, N=5.

Abdominal Aorta (3-day HU treatment)



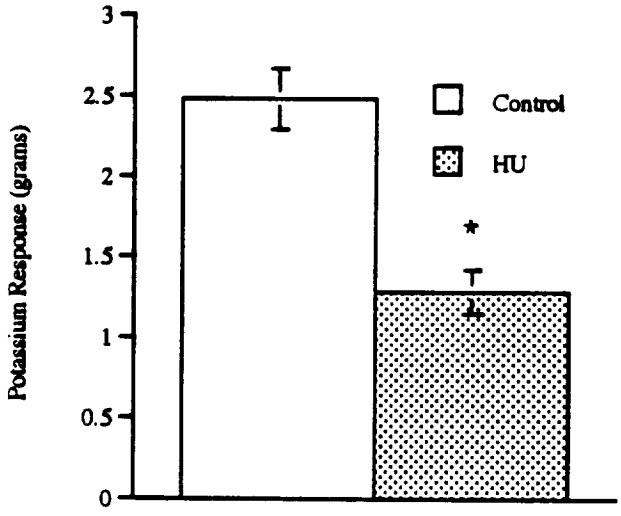


Figure 17. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in abdominal aorta from control and 3-day HU rats. *, $P<0.05$, N=5.

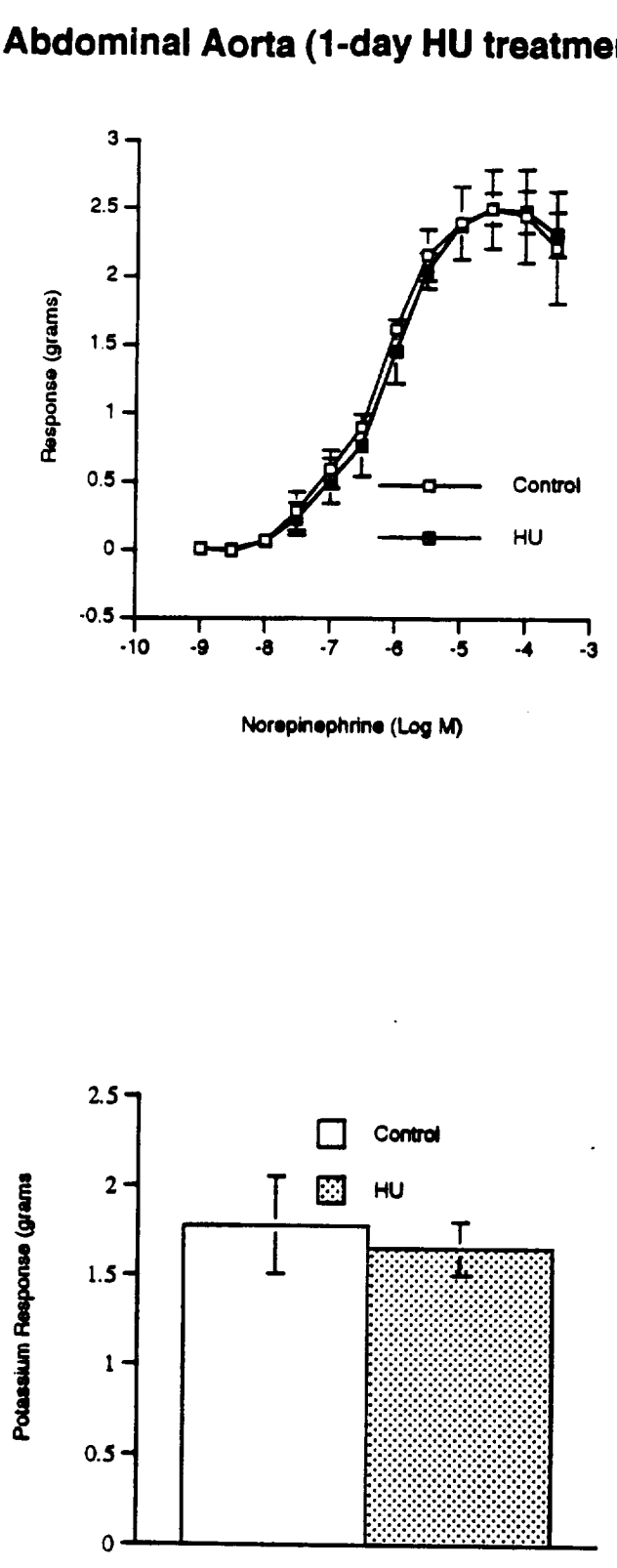


Figure 18. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in abdominal aorta from control and 1-day HU rats. *, $P<0.05$, N=5.

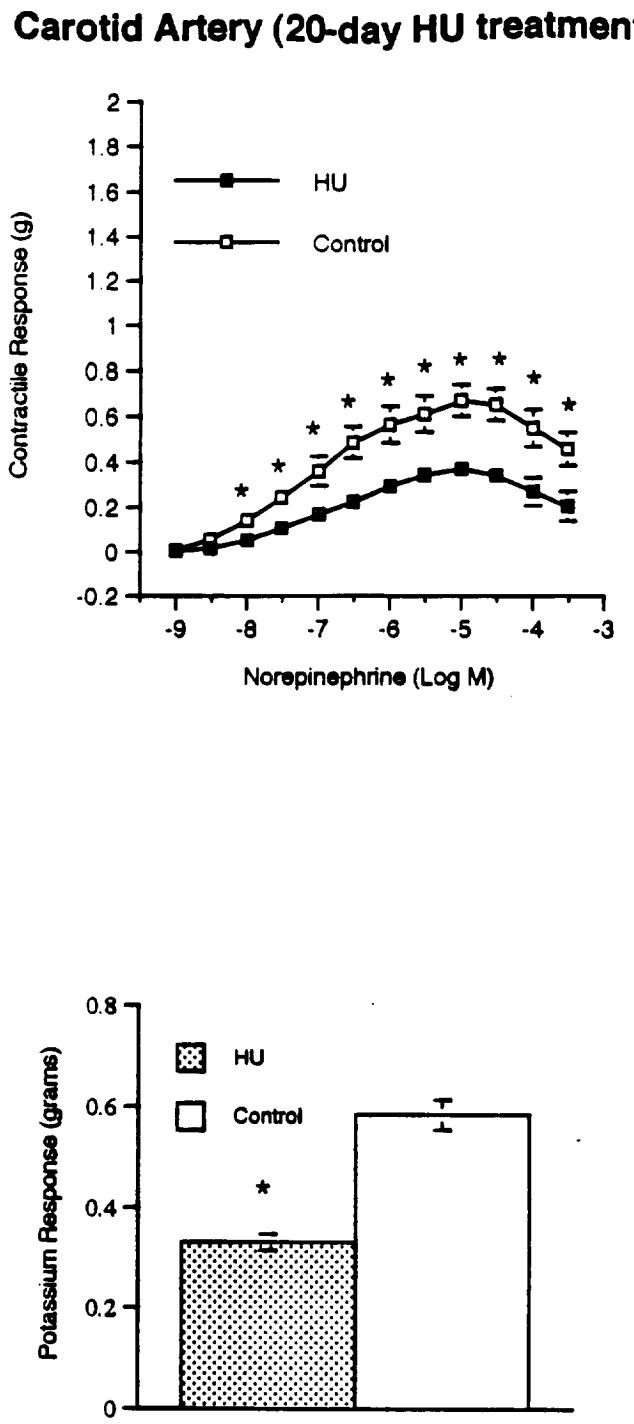


Figure 19. Concentration-response curves for the contractile effect of norepinephrine (N=6-7; upper panel), and the contractile response to 68 mM K^+ (N=4-6; lower panel) in carotid artery from control and 20-day HU rats. *, $P<0.05$.

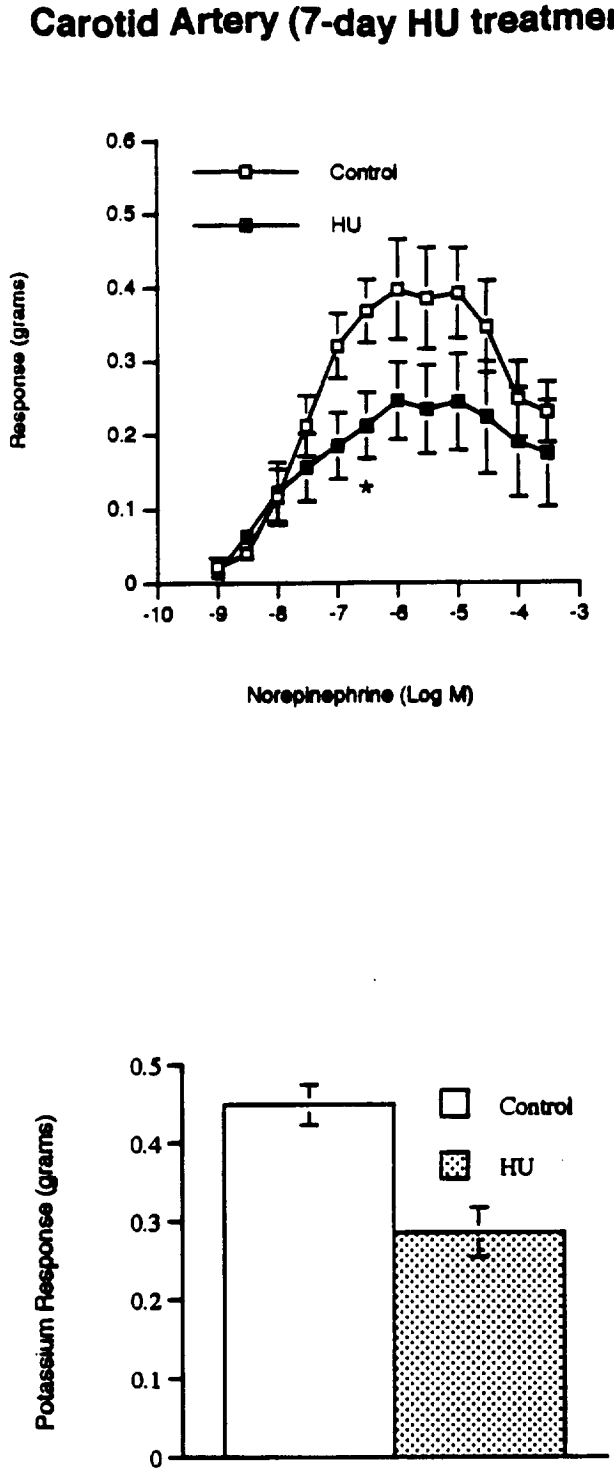


Figure 20. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in carotid artery from control and 7-day HU rats. *, $P<0.05$, $N=5$.

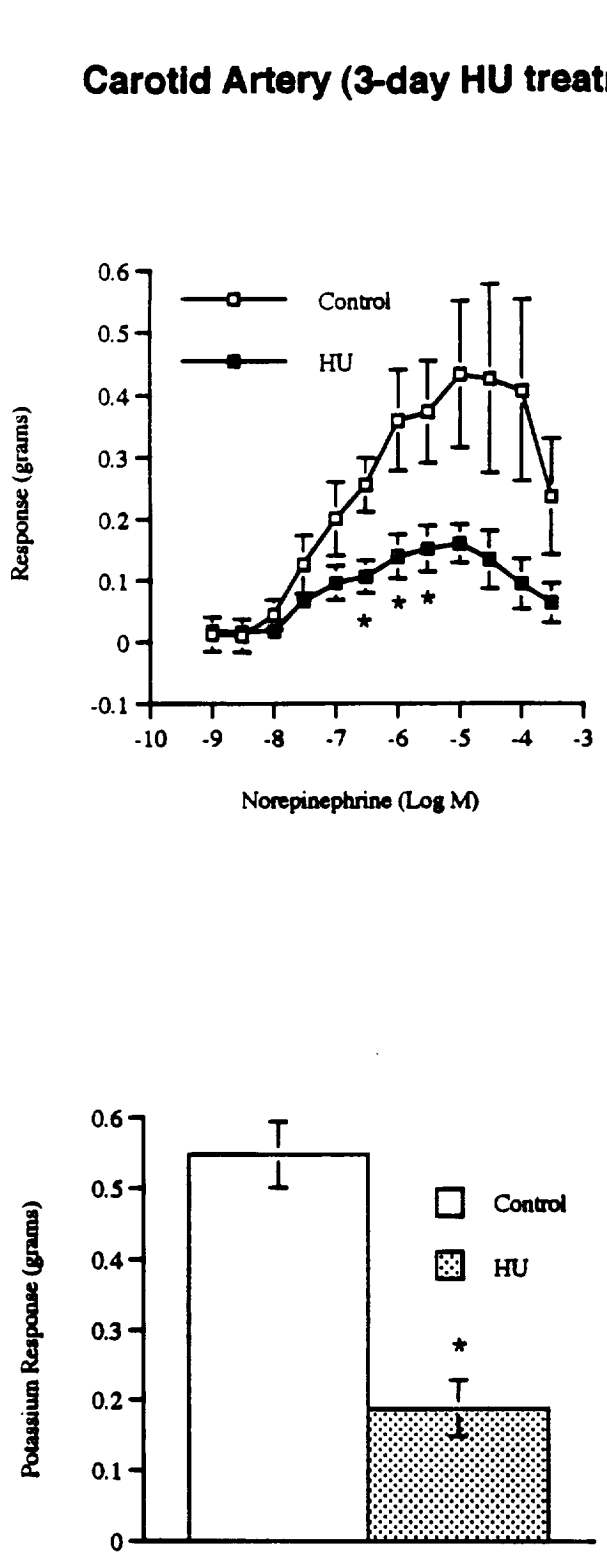


Figure 21. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in carotid artery from control and 3-day HU rats. *, $P<0.05$, N=5.

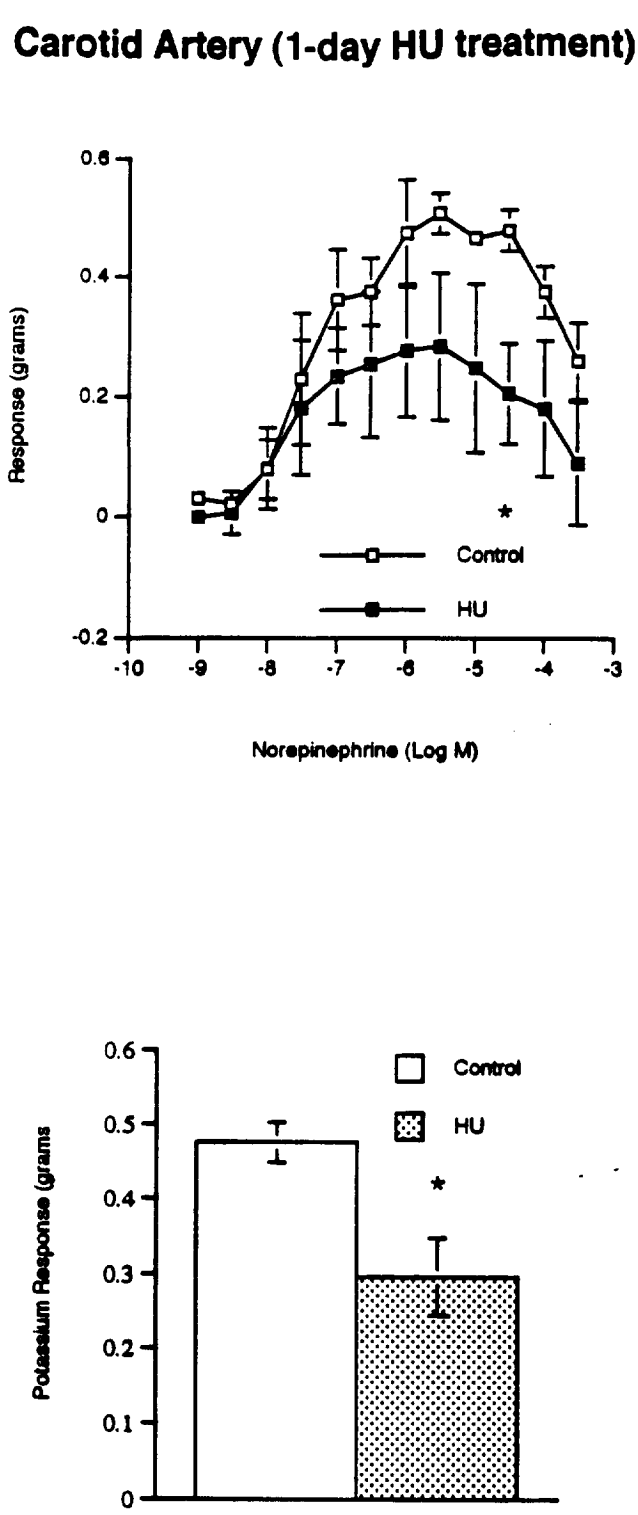


Figure 22. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in carotdi artery from control and 1-day HU rats. *, $P<0.05$, N=5.

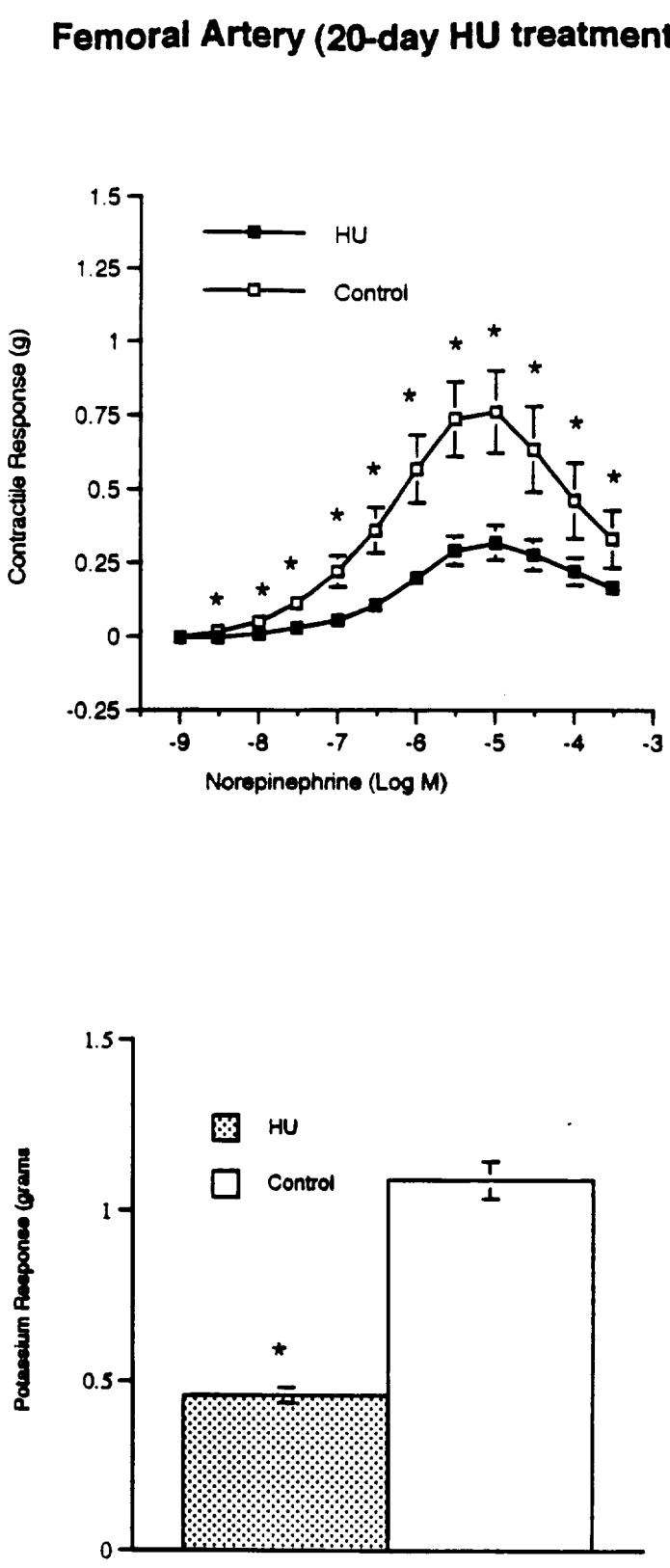


Figure 23. Concentration-response curves for the contractile effect of norepinephrine (N=4-6; upper panel), and the contractile response to 68 mM K^+ (N=4-6; lower panel) in femoral artery from control and 20-day HU rats. *, $P<0.05$.

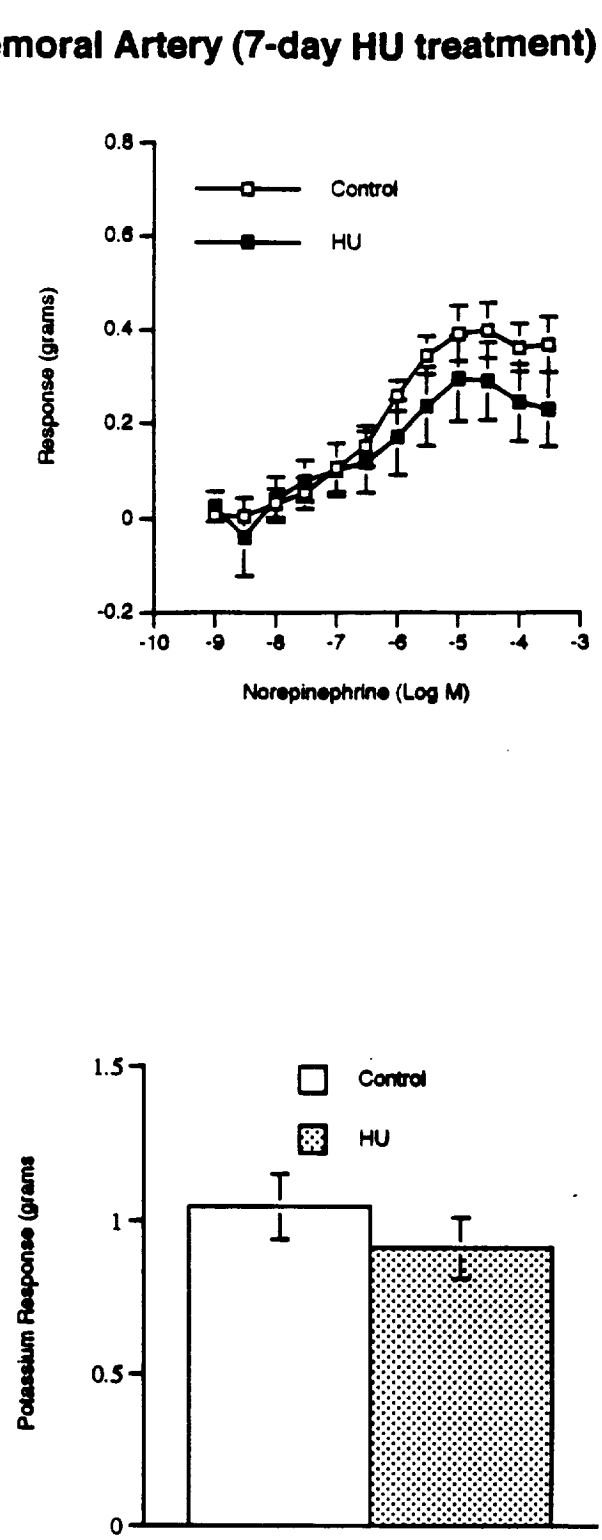
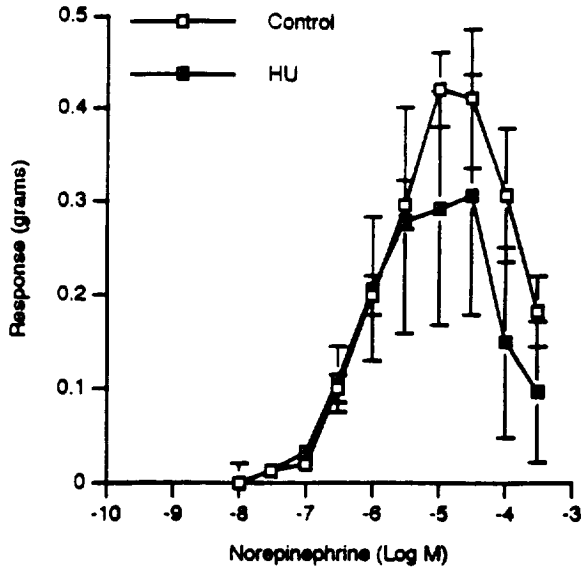


Figure 24. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) *in femoral artery* from control and 7-day HU rats. N=5.

Femoral Artery (3-day HU treatment)



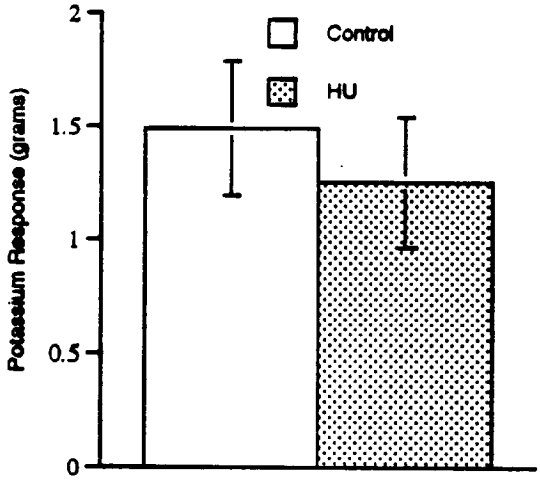


Figure 25. Concentration-response curves for the contractile effect of norepinephrine (upper panel), and the contractile response to 68 mM K^+ (lower panel) in femoral artery from control and 3-day HU rats. N=3.

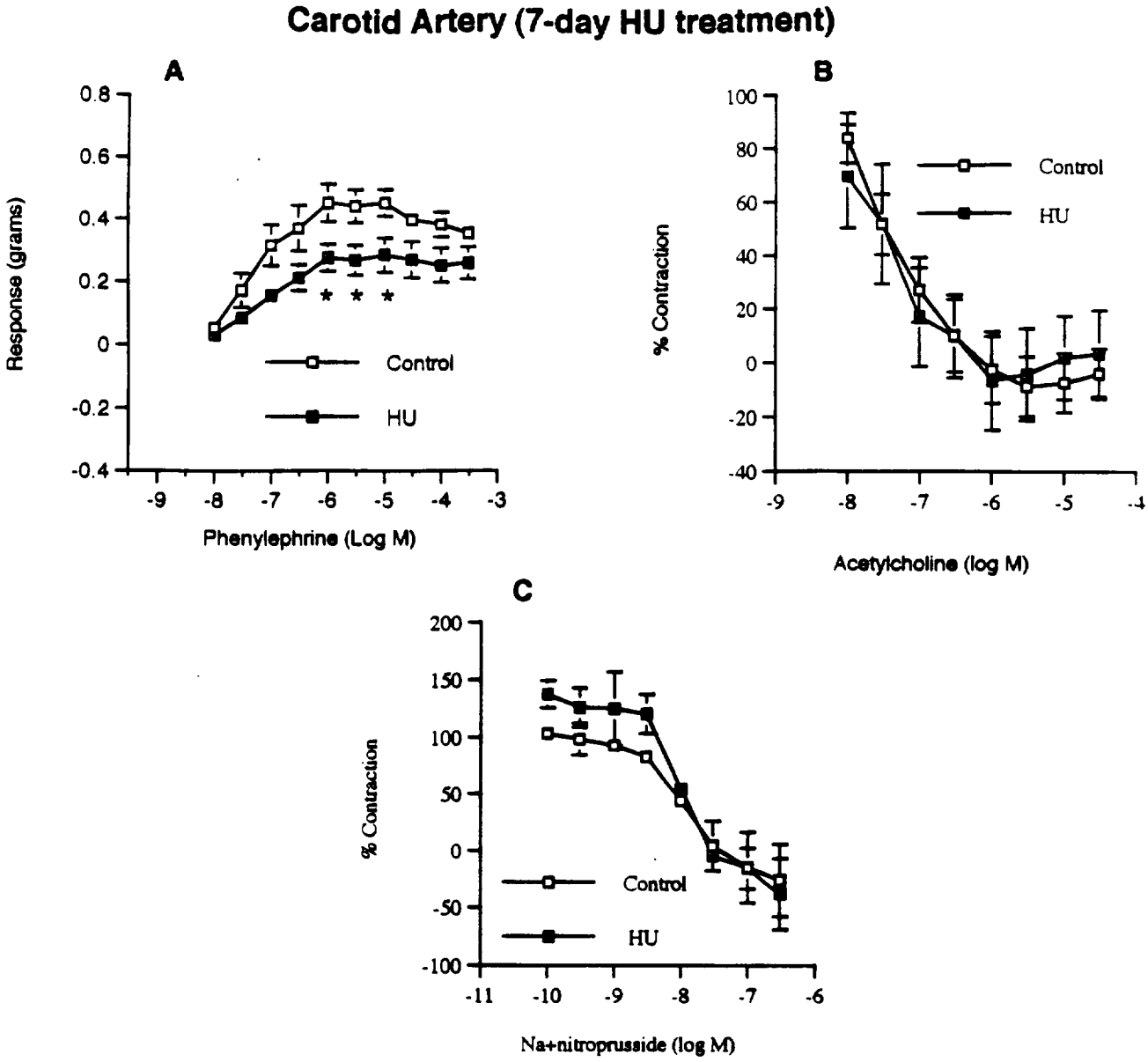


Figure 26. Concentration-response curves for the contractile effect of phenylephrine (panel A) and the relaxation effects of acetylcholine (panel B) and Na^+ nitroprusside (panel C) in carotid arteries from control and 7-day HU rats. *, $P<0.05$, $N=5$.

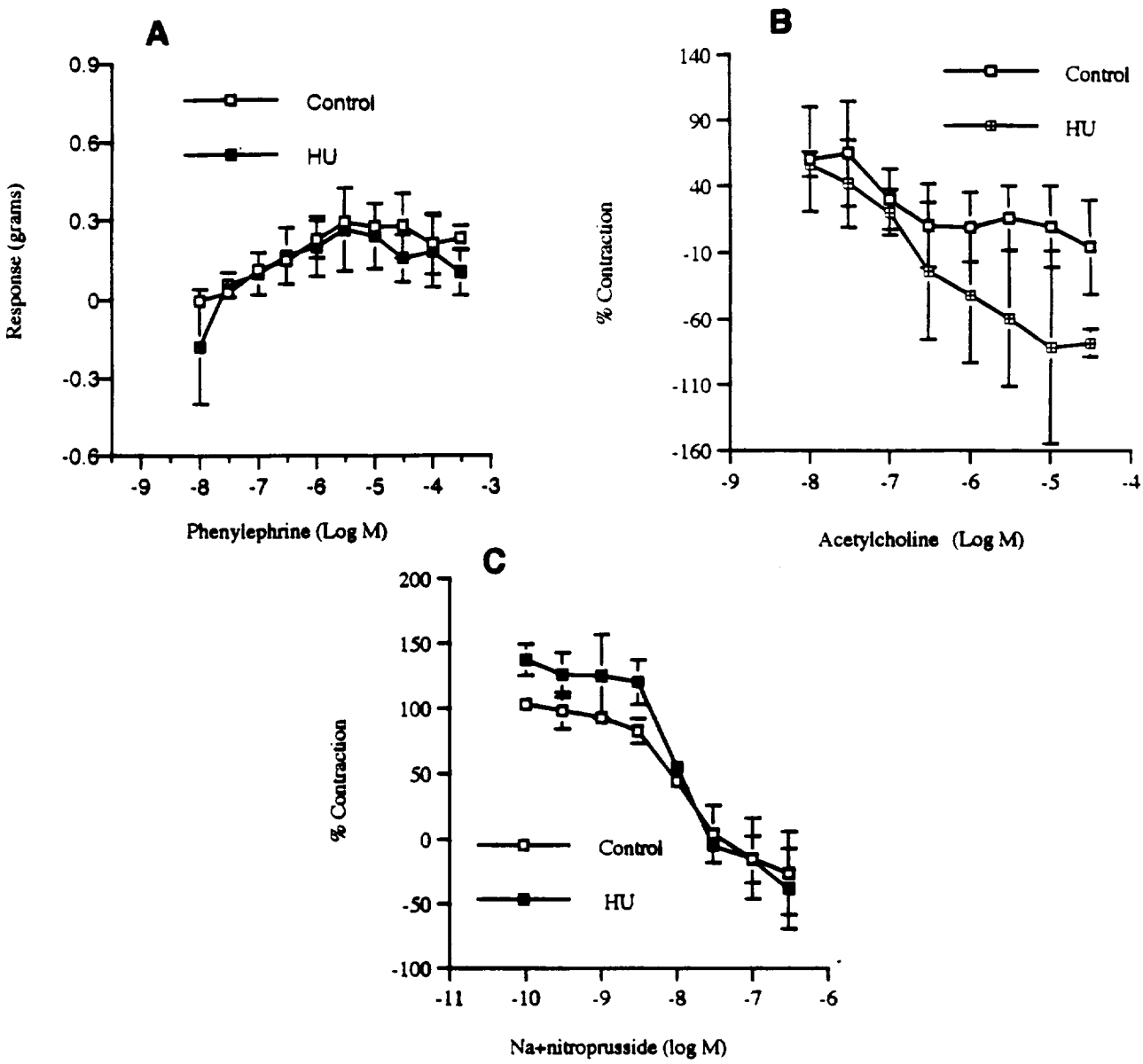


Figure 27. Concentration-response curves for the contractile effect of phenylephrine (panel A) and the relaxation effects of acetylcholine (panel B) and Na^+ nitroprusside (panel C) in carotid arteries from control and 20-day HU rats. N=2.

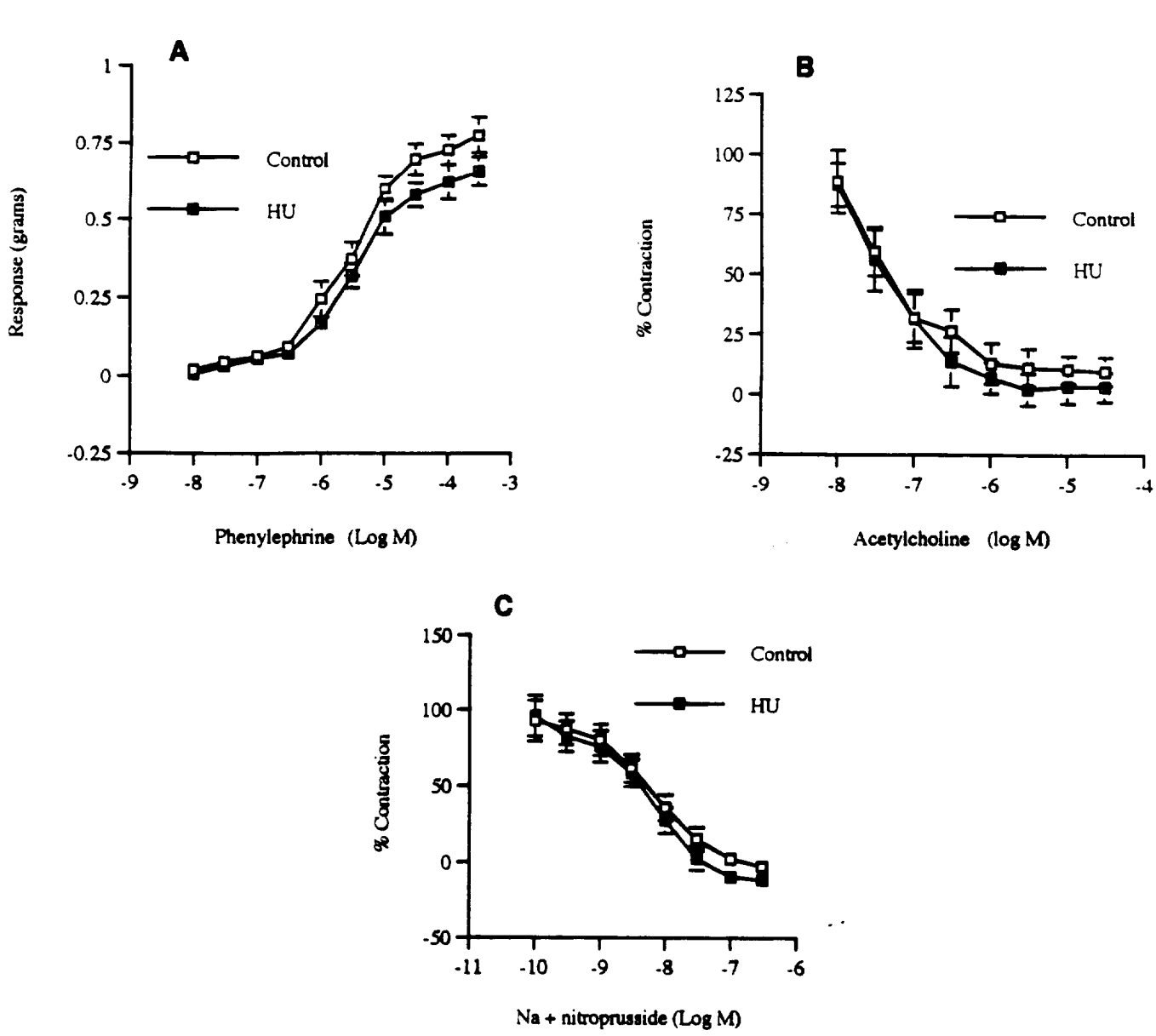


Figure 28. Concentration-response curves for the contractile effect of phenylephrine (panel A) and the relaxation effects of acetylcholine (panel B) and Na^+ nitroprusside (panel C) in femoral arteries from control and 20-day HU rats. *, $P<0.05$, N=5.